RESPIRATORY MEDICINE

In vitro expression of Fas and CD40 and induction of apoptosis in human cystic fibrosis airway epithelial cells

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Abstract Cystic fibrosis is characterized by a damaged airway epithelium with inflammation and chronic infection. The aim of this study was to investigate the process of apoptosis in this disease. To evaluate the effects of interferon γ and the Fas apoptotic pathway on cystic fibrosis airway epithelial cells, we used immortalized cystic fibrosis (CFT-1 and CFT-2) and normal (NT-1) human tracheal epithelial cell lines. Cell death was determined using annexin-V/propidium iodide labelling and electron microscopy. *In vitro* expression of Fas and CD40 surface antigens was analysed by immunofluorescence staining and flow cytometry. Normal and cystic fibrosis cells constitutively express these antigens. CD40, but not Fas expression, was upregulated by interferon γ. Treatment of interferon γ-stimulated cells with anti-Fas resulted in apoptosis for about 80% of CFT-2 (homozygous for ΔF508 deletion) cells and for 35–40% of CFT-1 (heterozygous) or normal cells. Our results suggest that Fas may mediate apoptosis in cystic fibrosis airway epithelium.

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (1). It results in absent or deficient expression of CFTR protein (2). The most common mutation is the ΔF508 mutation which has a worldwide prevalence of about 70% of CF chromosomes in caucasians (3). CFTR protein is expressed in epithelial cells of the pancreas, intestine, sweat glands, upper and lower expiratory tracts. The main cause of morbidity and mortality in CF is linked to chronic inflammatory and infectious bronchial processes which lead to progressive bronchial damage and lung destruction (4). CFTR protein is a cAMP-regulated chloride channel and defective electrolyte transport is believed to be the initial abnormality in CF disease, resulting in alteration of epithelial secretions (5). Evidence of an intense and sustained airway inflammation has been reported in several studies (6). A possible role of CFTR dysfunction in entertaining inflammatory and infectious processes through a dysregulation of epithelial ion transport, abnormal mucociliary clearance and cytokine production, has been suggested (7).

Programmed cell death or apoptosis is a physiological process defined by distinct characteristic morphological and biochemical changes (8). It is essential for the maintenance of homeostasis of epithelial organisation and function and for clearance of inflammatory cells (9). It is regulated by several factors, including oxidative stress, extracellular matrix proteins, and external signals as tumour necrosis factor (TNF)α, CD40 or Fas ligation (10–12). Fas (CD95) and CD40, two molecules of the plasma membrane, are involved in the regulation of apoptosis in different cellular models, such as lymphocytes (11,12). CD40, a molecule belonging to the superfamily of the TNF receptor, was initially detected in B lymphocytes (13). CD40 signaling does not always deliver an anti-apoptotic activation (14) and may instead lead to apoptosis (15–17). Fas antigen (CD95, APO-1), another member of the TNF receptor family (18), induces apoptosis after engagement with the Fas ligand (19). Several recent studies have demonstrated that interferon γ (IFNγ), alone or in combination with activation of the Fas pathway, induces apoptosis in various Fas-expressing cells, such as cultured keratinocytes (20), breast cell lines (21), and lung epithelial cells (22). There are some discrepancies

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concerning the interaction between CD40 and Fas to enhance or inhibit apoptosis (16,23).

Apoptosis dysfunction in CF has recently been suggested, but with discordant results. A "resistance" in the initiation of apoptosis induced by etoposide was reported in a mutant ΔF508 epithelial mouse mammary cell line, probably due to the inability to obtain cytoplasmic acidification (24). On the other hand, a high DNA fragmentation and a high level of Fas ligand expression have been reported in both human CF enterocytes and bronchial epithelial cells (25,26)

The purpose of this study was to investigate CD40 and Fas expression in human CF and non-CF tracheal epithelial cell lines and compare the susceptibility of these cell lines to Fas-mediated apoptosis induced by anti-Fas monoclonal antibody with or without IFNγ.

MATERIALS AND METHODS

Human tracheal epithelial cell cultures

Transformed tracheal cell lines were used because acceptable quantitative and qualitative primary CF epithelial cells are difficult to obtain in acceptable quality and quantity in vitro. Three human SV40-transformed tracheal epithelial cell lines were kindly provided by A. Paul (INSERM U402, Paris, France). NT-1 cell line deriving from non-CF human foetus served as control. The two CF cell lines used in this study were obtained from CF foetuses with different mutations in the CFTR gene: CFT-1 cells which are compound heterozygote for S549N and N1303K mutations (class III mutations) and CFT-2 cells which are homozygous for the ΔF508 mutation (class II mutation) (27).

Cells were grown in a 1:1 ratio mixture of Dulbecco's minimum essential medium and Ham's F12 (DMEM/F12, Gibco) nutrient medium supplemented with 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin, 250 ng ml^{-1} of amphotericin B and 10% of foetal calf serum (Gibco). Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C until sub-confluence and the medium was changed three times weekly.

Immunofluorescence staining and flow cytometric analysis

Before reaching sub-confluence, human tracheal epithelial monolayers were pretreated for 48 h with TNFα (100 U ml^{-1}, Sigma) or with IFNγ (1000 U ml^{-1}, Genzyme) prior to immunofluorescence staining. Cells left untreated in culture medium were used as controls. At sub-confluence, adherent epithelial cells were detached by incubation with EDTA (0·2 g l^{-1}) for 2–3 min.

Surface antigen expression was then performed at 4°C with CD40 (mAb 89, 10 μg ml^{-1}, generously provided by Schering-Plough, Dardilly, France) and Fas (UB2, 5 μg ml^{-1}, Immunotech).

R-phycoerythrin-conjugated anti-mouse immunoglobulin (Ig) alone as well as irrelevant isotype-matched mouse anti-human IgG1 served as negative controls. Fluorescence intensity of at least 5×10^3 cells per sample was analysed using fluorescence-activated cell sorter (FACS EPICS XL-MCL). The percentage of labelled cells was evaluated and the mean fluorescence intensities (MFI) were determined. Statistical analysis of at least six different experiments for each cell line was performed with Wilcoxon matched-pair signed-rank test.

Induction of apoptosis by anti-Fas antibody and cell death assay

At semiconfluence, cells were either pre-treated or not with recombinant human IFNγ (1000 U ml^{-1}) for 24 h. Tracheal epithelial cells were then washed and either treated or not with anti-Fas mAb (IgM, CH-11, 0·1 and 1 μg ml^{-1}, Immunotech) for an additional 48 h culture. At this time, cells were sub-confluent except for those incubated with IFNγ and/or anti-Fas, some of which had detached from the culture surface. Adherent and nonadherent cells were then harvested, adherent cells being removed by incubation in trypsin/EDTA whereas nonadherent cells, detached during the last 48 h period, were recovered by collecting the culture medium.

After the washing steps, viability and numeration of cells were determined by trypan blue exclusion.

Apoptosis was assayed with the Annexin-V-FLUOS Staining Kit from Boehringer Mannheim, according to the manufacturer's protocol. Annexin-V-FLUOS binds specifically to phosphatidylserine exposed after translocation on the outer membrane of apoptotic cells but cannot react with the inner membrane phosphatidylserine of normal cells. Briefly, cell suspensions were incubated with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide for 10–15 min at 4°C and analysed by FACS as rapidly as possible.

To differentiate necrotic from apoptotic cells and to detect ultrastructural apoptotic changes, electron microscopy was performed on epithelial cells under the same conditions as IFNγ and/or anti-Fas treatment. Adherent cells and supernatants were sequentially fixed with 2% glutaraldehyde in culture medium at room temperature for 15 min (2% glutaraldehyde in 0·1 M Na cacodylate-HCl, pH 7·4 at room temperature, for 15 min), washed three times in cacodylate buffer–0·2 M sucrose and post-fixed with 1% OsO4–0·15 M Na cacodylate-HCl (4°C, 30 min). Cells were then contrasted *in situ* with 3% aqueous uranyl acetate (room temperature, 45 min). After dehydration in graded ethanol, the cell layer was embedded in epon, polymerized (60°C, 48 h) and horizontally sectioned with LKB Ultrotome V. Ultrathin

sections (60–80 nm) were observed with JEOL 1200 EX transmission electron microscope.

RESULTS

Antigen expression in vitro

Results of antigen expression analysis by flow cytometry are summarized in Fig. 1. Fas and CD40 molecules are constitutively expressed by the three cell lines.

Fas expression was not significantly modified after cytokine treatment. A small increase in the percentage of positive cells was only observed after IFNγ stimulation of NT-1 cells. IFNγ up-regulated CD40 expression and this increase was significant in cystic fibrosis cells. Stimulation with TNFα only enhanced the number of stained cells for the homozygous CF cell line (CFT-2).

Fas-induced apoptosis data

To determine whether the three cell lines were susceptible to Fas-mediated apoptosis, Fas mAb was applied to the cultures during 48 h. We investigated the presence of apoptotic cells using newly described fluorescence

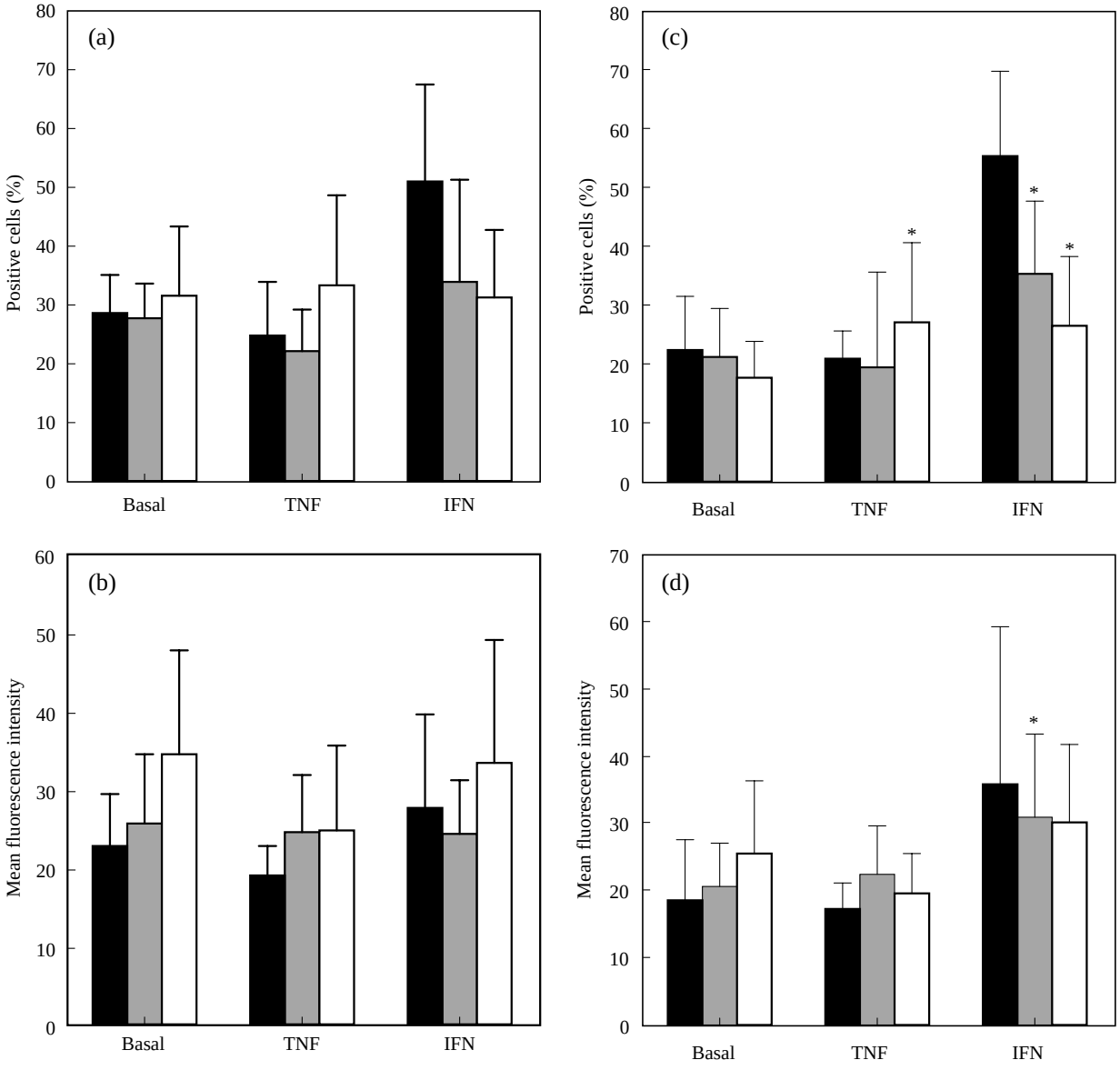

FIG. 1. Fas (a,b) and CD40 (c,d) expression in tracheal epithelial cell lines. Modulation by pro-inflammatory cytokines. Presented are bar graphs representing percentages of positive cells (a,c) and corrected surface antigen mean fluorescence (MFI, b,d). The three cell lines (NT-1, ■; CFT-1, ▨ and CFT-2, □;) were either left untreated (basal conditions) or treated during 48 h with TNFα (100 U ml^{-1}) or IFNγ (1000 U ml^{-1}). Presented results are the means ± SD of 6–10 experiments. *$P < 0{\cdot}05$ when compared with basal conditions (statistical analysis performed with Wilcoxon matched-pair signed-rank test).

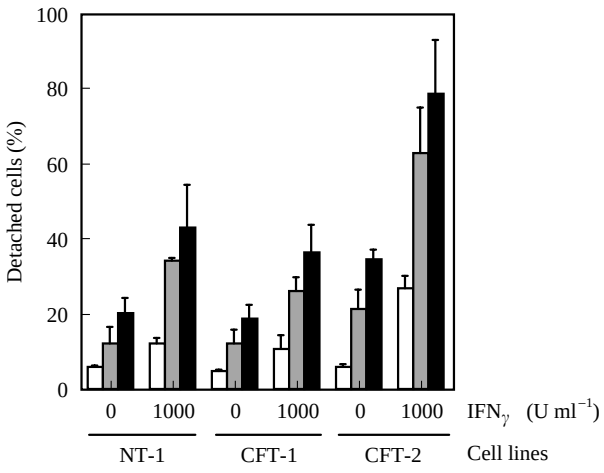

FIG. 2. Cell detachment induced by anti-Fas antibody. Tracheal epithelial cells from each cell line, either pre-treated or not with IFNγ for 24 h, were cultured with 0 (□), 0·1 (▨) or 1 (■) μg ml^{-1} of anti-Fas mAb for an additional 48 h period. Non-adherent cells, detached during this last period, were recovered by collecting the culture medium and numerated afterwards. The figure shows the results (mean ± SD) for three independent experiments.

methods by exposure to PI and Annexin V labelling. Results were obtained for three experiments. Fas mAb induced detachment of the cells in the three cell lines and, in all cases, more than 90% of the detached cells expressed both Annexin V and PI, corresponding to advanced apoptotic cells and necrotic cells. However, the number of detached cells varied significantly between the three cell lines as shown in Fig. 2. Treatment alone (1 μg ml^{-1}) induced detachment of 20% of control cells (NT-1), 19% of heterozygous CF cells (CFT-1), and 35% of homozygous CF cells (CFT-2). With a lower dose of anti-Fas (0·1 μg ml^{-1}), a similar but less pronounced effect was obtained in all the cell lines (12, 12 and 21·5%, respectively). Very few detached cells (5–6%) were detected under basal conditions. Preincubation with IFNγ for 24 h enhanced the effect of anti-Fas mAb (two to three-fold increase of detachment). Treatment with IFNγ alone also induced detachment in 11–12% of NT-1 and CFT-1 cells and 27% of CFT-2 cells. Electron microscopic findings (Fig. 3) confirmed the presence of apoptotic rather than necrotic nuclei in tracheal epithelial cells treated with anti-Fas antibody. Chromatin condensation and cytoplasmic vacuoles, which are typical features of apoptosis, were observed in most detached CF [Fig. 3(a–c)] and control (data not shown) cells. On the other hand, adherent cells did not show apoptotic nuclear changes [Fig. 3(d)].

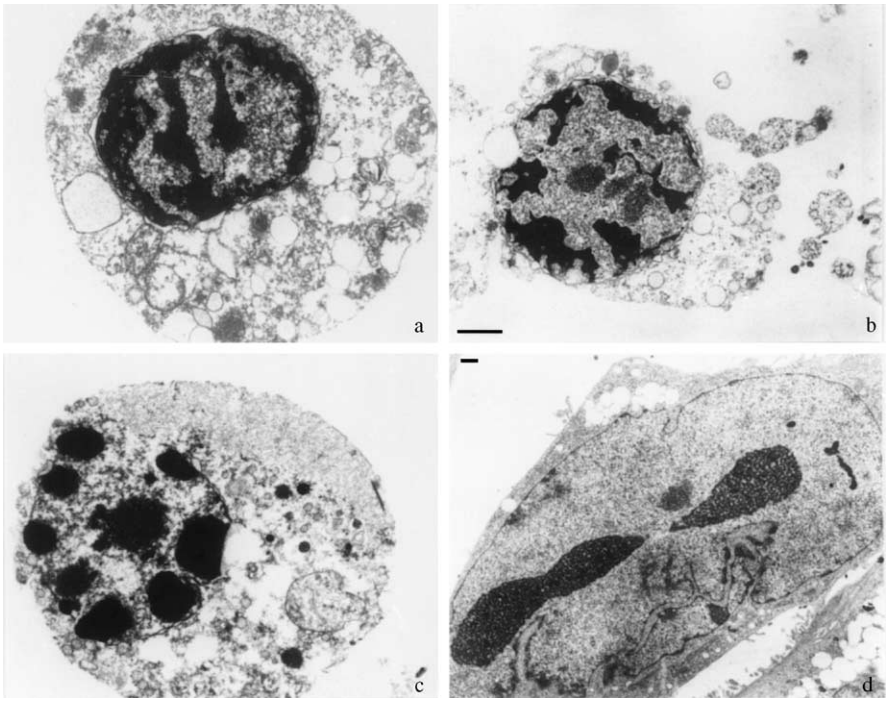

FIG. 3. Ultrastructure of apoptosis in human epithelial tracheal cell lines. (a, b, c) Detached cystic fibrosis tracheal cells. CFT-1 (a, b) and CFT-2 (c) cells treated with IFNγ+1 μg ml^{-1} of anti-Fas antibody. Condensed perinuclear chromatin is observed, cytoplasmic and nuclear membrane integrity is preserved but cytomembranes are vesiculated (a, c). Numerous nuclear apoptotic masses can be distinguished from the nucleoli (b) (bar = 1 μm). (d) Adherent non apoptotic control cells (NT-1, non-CF cell treated with IFNγ+1 μg ml^{-1} of anti-Fas antibody). The cell is widely spread. The cytoplasmic organelles are intact, discrete chromatin condensations are scattered along the nuclear envelope contrasting with large typical nucleoli (bar = 1 μm).

In adherent cells, the percentage of cells expressing annexin and/or PI did not vary whatever the cell type or treatment.

DISCUSSION

Our study reveals that both CF and control cultured human tracheal epithelial cells constitutively express Fas and CD40 antigens with an upregulation of CD40 expression by IFNγ in the three lines. We have recently shown that both deficient and normal CFTR genotype cell lines also express Fas receptor ligand (FasL) with a higher mean fluorescence intensity in CF genotype cells (26). As these surface antigens are potentially involved in the apoptotic process, they could play a role in controlling epithelial cell turnover. Coexpression of Fas/FasL has been demonstrated in human airway epithelial cells deriving from primary cultures, immortalized cell lines (28) and in bronchial biopsies from healthy (28, 29), asthmatic (29) and CF (26) subjects, but with a higher level of FasL expression in asthmatic and CF patients as compared with controls (26, 29). The authors suggested that dysregulation of the Fas/FasL interaction could induce self or adjacent-cell death, which should result in a high cell turnover.

The present study also demonstrated that anti-Fas monoclonal antibody induced apoptosis of human tracheal epithelial cells *in vitro* in a dose-dependent manner. Airway epithelial cells from the three cell lines were more susceptible to anti-Fas after treatment with IFNγ. This phenomenom, demonstrating that IFNγ acts in synergy with the Fas apoptotic pathway, was also observed in keratinocytes (20) and lung epithelial cells (22). This could suggest a role for the up-regulation of CD40 expression by IFNγ in the enhanced apoptosis.

The apoptotic function of TNFα did not seem to be dependent on Fas expression or the Fas pathway.

Interestingly, whereas Fas was similarly expressed in the three cell types, Fas-induced apoptosis was markedly increased in homozygous ΔF508/ΔF508 CFT-2 cells (about a two-fold increase) as compared with control NT-1 cells and heterozygous CFT-1 cells. Fas expression was not significantly modified by IFNγ treatment, but preincubation of cells with IFNγ enhanced CFT-2 anti-Fas cell death induction.

Discrepancies observed in susceptibility to apoptosis between CFT-1 and CFT-2 still remain to be clarified. One hypothesis could be that, in the case of homozygous ΔF508/ΔF508 CFT-2 cells, there is an accumulation of the CFTR mutant protein in the endoplasmic reticulum that could induce activation of transcriptional factors, such as nuclear factor κB(NF-κB), which plays an important role in apoptosis and in inducing Fas ligand expression (30).

Resistance to induction of apoptosis has been demonstrated in mouse mammary CF epithelial cells which do not express the apical CFTR (24). The 'authors' hypothesis was the inhability of CF cells to obtain cytoplasmic acidification. It has been demonstrated that ΔF508/ΔF508 CF cells—which do not express CFTR protein at the apical membrane because of accumulation in the endoplasmic reticulum—present a defect in acidification (31). However, in some cell-free models, a pH above 7·4 only suppressed the activity of procaspase-3 but not the activity of caspase-3, which is a final common pathway for most apoptotic signals (32). This suggests that pH and salt could affect activation of apoptosis by different pathways and in different ways. The use of different mediators (cycloheximide or etoposide) to induce cell death could explain the apparent discrepancy with our results. Furthermore, we do not have information about Fas expression and susceptibility to anti-Fas antibody in this mouse mammary cell line.

In conclusion, ΔF508 homozygous human tracheal cells have high susceptibility to Fas-mediated apoptosis, especially with IFNγ pretreatment. Whether this result is related to the high level of FasL expression remains to be confirmed. The role of enhanced Fas mediated apoptosis in the dysregulation of inflammatory and fibrotic epithelial process observed in CF could be suggested, but further studies are required.

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